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Published in:
Archives of Microbiology

DOI:
10.1007/s002030100270

Publication date:
2001

Arch Microbiol (2001) 175 : 334–343
DOI 10.1007/s002030100270

ORIGINAL PAPER

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Genetic and functional characterization of *dpp* genes encoding a dipeptide transport system in *Lactococcus lactis*

Received: 14 November 2000 / Revised: 22 February 2001 / Accepted: 28 February 2001 / Published online: 19 April 2001

Abstract The genes encoding a binding-protein-dependent ABC transporter for dipeptides (Dpp) were identified in *Lactococcus lactis* subsp. *cremoris* MG1363. Two (*dppA* and *dppP*) of the six ORFs (*dppAdppPBCDF*) encode proteins that are homologous to peptide- and pheromone-binding proteins. The *dppP* gene contains a chain-terminating nonsense mutation and a frame-shift that may impair its function. The functionality of the *dpp* genes was proven by the construction of disruption mutants via homologous recombination. The expression of DppA and various other components of the proteolytic system was studied in synthetic and peptide-rich media and by using isogenic peptide-transport mutants that are defective in one or more systems (Opp, DtpT, and/or Dpp). In peptide-rich medium, DppA was maximally expressed in mutants lacking Opp and DtpT. DppA expression also depended on the growth phase and was repressed by tri-leucine and tri-valine. The effect of tri-leucine on DppA expression was abolished when leucine was present in the medium. Importantly, the Dpp system also regulated the expression of other components of the proteolytic system. This regulation was achieved via the internalization of di-valine, which caused a 30–50% inhibition in the expression of the proteinase PrtP and the peptidases PepN and PepC. Similar to the regulation of DppA, the repressing effect was no longer observed when high concentrations of valine were present. The intricate regulation of the components of the proteolytic system by peptides and amino acids is discussed in the light of the new and published data.

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Introduction

In bacteria, a large fraction of the nutrient uptake capacity is mediated by members of the ABC superfamily of transporters. These are multicomponent systems consisting of two integral membrane subunits and two peripheral ATP-binding subunits, which function together with an extracytoplasmic solute-binding receptor (Saier 1998). ABC transporters may have a role not only in nutrient accumulation, but also in other cellular processes (Lazazzera et al. 1998). Regulation of gene expression is achieved when the translocated solute serves as a signaling molecule that interacts with an intracellular receptor, or when the transporter transduces a signal across the cytoplasmic membrane upon ligand binding (Leskelä et al. 1999). The role of peptide transport systems in functions other than nutrition is exemplified by the oligopeptide permease (Opp) of *Bacillus subtilis*, which is involved in competence development and sporulation (Perego et al. 1991; Rudner et al. 1991), or the dipeptide permease (Dpp) of *Escherichia coli*, which is involved in chemotaxis (Abouhamad et al. 1991; Manson et al. 1986).

The utilization of milk proteins by lactococci requires the coordinate action of a set of proteolytic enzymes and peptide-transport systems. In *Lactococcus lactis*, the secondary transporter for di- and tripeptides (DtpT) and the ABC transporter for oligopeptides (Opp) are well-characterized (Detmers et. al. 1998; Hagting et al. 1994; Lanfermeijer et al. 1999; Picón et al. 2000). Most of the casein-derived peptides (>98%) initially generated by the extracellular serine proteinase PrtP consist of 5–26 residues, and these are possible substrates for the Opp system (Kunji et al. 1996). The di- and tripeptide transporters are thought

to play an important role in regulation or signaling processes. Several studies of the regulation of components of the proteolytic system have already revealed control of gene expression by the nitrogen source (Marugg et al. 1995; Meijer et al. 1996). Thus, the presence of specific peptides, such as Leu-Pro or Pro-Leu, internalized via DtpT, down-regulates the expression of PrtP, PrtM, PepN, and PepX. Recent studies also indicate that peptides consisting of hydrophobic branched-chain amino acids regulate transcription of different components of the proteolytic system (Guédon and Renault, INRA, Jouy-en-Josas, unpublished data). In *L. lactis*, the existence of at least a third peptide transport system has been inferred from the residual ability of mutants lacking the DtpT and Opp systems to utilize di- and tripeptides of hydrophobic nature (Foucaud et al. 1995). In this work, the genes coding for a putative ABC dipeptide transporter (Dpp) in *L. lactis* subsp. *cremoris* MG1363 were identified. The genomic organization of the *dpp* operon and the functional characterization of the gene products are presented. The roles of *dpp* in dipeptide utilization and regulation of the expression of proteolytic enzymes are discussed.

Table 1 Strains and plasmids

Strains/plasmids	Relevant properties	Reference or source
Strains		
L. lactis		
MG1363	Opp^+, $DtpT^+$, Dpp^+, Lac^-, Prt^-; plasmid-free NCDO712	Gasson (1983)
AG300	Opp^+, $DtpT^-$, Dpp^+, Lac^-, Prt^-; MG1363, Δ*dtpT*	Hagting et al. (1994)
AG500	Opp^-, $DtpT^-$, Dpp^+; MG1363 Δ*pepO*, Δ*dtpT*, Δ*opp*	Hagting et al. (1994); Kunji et al. (1996)
LL108	Cm^r, $RepA^+$; MG1363 carrying the pWV01 *repA* gene integrated in the chromosome	Leenhouts et al. (1996)
YS5A	Opp^-, $DtpT^-$, Dpp^-, Em^r; AG500 *dppA*::pINTdppA	This work
YS5B	Opp^-, $DtpT^-$, Dpp^-, Em^r; AG500 *dppB*::pINTdppB	This work
YS5F	Opp^-, $DtpT^-$, Dpp^-, Em^r; AG500 *dppF*::pINTdppF	This work
YS5BP	Opp^-, $DtpT^-$, Dpp^-, Em^r, Lac^+, Prt^+; YS5B (pLP712)	This work
MGE	Opp^+, $DtpT^+$, Dpp^+, Lac^-, Prt^-, Em^r; MG1363 (pAMP0)	This work
AG5E	Opp^-, $DtpT^-$, Dpp^+, Em^r; MG1363 Δ*pepO*, Δ*dtpT*, Δ*opp* (pAMP0)	This work
AG5EP	Opp^-, $DtpT^-$, Dpp^+, Lac^+, Prt^+, Em^r; MG1363 Δ*pepO*, Δ*dtpT*, Δ*opp* (pAMP0, pLP712)	This work
NZ9000	MG1366, *pepN*::*nisRK*	Kuipers, University of Groningen, The Netherlands, unpublished data
NZ9700	Nisin-producing transconjugant containing Tn*5276*	Kuipers et al. (1993)
Plasmids		
pLP712	Lac^+, Prt^+, conjugative plasmid of NCDO712 containing the *lac* and *prtP/prtM* genes	Gasson (1983)
pORI28	Em^r, Ori^+, $RepA^-$, pWV01-derivative, only replicates in strains providing *repA* in *trans*	Leenhouts and Venema (1993)
pINTdppA	Em^r, pORI28-derivative carrying a 0.86-kb fragment of *dppA*	This work
pINTdppB	Em^r, pORI28-derivative carrying a 0.5-kb fragment of *dppB*	This work
pAMP0	Em^r, pAMP31-derivative, empty vector	Picón et al. (2000)
pINTdppF	Em^r, pORI28-derivative carrying a 0.65-kb fragment of *dppF*	This work
pHLP5	Cm^r, pNZ8048-derivative carrying *lmrP* with 3'-end His-tag sequence	Putman et al. (1999)
pNZCIP	Cm^r, pNZ8048-derivative carrying *citP* with 5'-end His-tag sequence	Bandell and Lolkema, University of Groningen, The Netherlands, unpublished data
pNZAE	Cm^r, pHLP5-derivative carrying 1.9-kb chromosomal fragment containing part of *dppA*	This work
pNZPE	Cm^r, pHLP5-derivative carrying 1.8-kb chromosomal fragment containing *dppP*	This work
pNZDppA	Cm^r, pHLP5-derivative carrying *dppA* without signal peptide sequence	Sanz et al. (2000)
pNZDppAL	Cm^r, pHLP5-derivative carrying *dppA*	This work
pNZDppP	Cm^r, pHLP5-derivative carrying *dppP* without signal peptide sequence	This work
pNZDppPL	Cm^r, pHLP5-derivative carrying *dppP*	This work

Materials and methods

Strains and growth conditions

The strains used in this study are listed in Table 1. Unless specified otherwise, *L. lactis* strains were grown at 30 °C in M17 broth or agar (Difco, East Molesey, UK) supplemented with 0.5% (w/v) glucose. Chloramphenicol or erythromycin was added to 5 μg/ml, when appropriate. To determine the ability of the strains to utilize peptides as a source of essential amino acids, the cells were grown in chemically defined medium (CDM, Poolman and Konings 1988). For these experiments, cells were grown in CDM until an OD_{660} of about 0.8, harvested by centrifugation (5,000×*g*, 10 min), and washed with sterile 25 mM sodium phosphate, pH 6.4. The cells were diluted to a final OD_{660} of 0.025 in fresh CDM containing all amino acids except an essential one that was supplied in the form of a di- or tripeptide. Growth rates were determined by monitoring the OD_{620} every 15 min in a SPECTRAmax 340 (Molecular Devices). Growth experiments were carried out in quadruplicate. Data were analyzed with a modification of the Gompertz equation (Zwietering et al. 1990). Non-linear least-square regression was performed with the Sigma Plot program (Jandel Scientific Software).

General DNA manipulations

Molecular cloning techniques were carried out essentially as described by Sambrook et al. (1989). Chromosomal DNA of *L. lactis* was isolated as previously described by Leenhouts et al. (1990). Plasmid DNA was isolated by either the method of Birnboim and Doly (1979), with minor modifications (Leenhouts et al. 1990), or the Qiagen column purification kit (Qiagen, Hilden, Germany). PCR was done with *Vent* (New England BioLabs, Beverly, Mass.) or *Taq* DNA polymerase (Boehringer, Almere, The Netherlands) according to the instructions ofthe suppliers. PCR products were purified with the Qiagen purification kit. Restriction endonucleases and T4 DNA ligase were used according to the instructions of the supplier (Boehringer). *L. lactis* was transformed by electroporation as described by Holo and Nes (1989).

Sequencing analyses

Diagnostic sequences in the genome of *L. lactis* subsp. *lactis* IL1403 were used to design primers to amplify the *dpp* genes and flanking regions of *L. lactis* subsp. *cremoris* MG1363 (Table 2). The sequence upstream of *dppA* and the 5′-end of this gene (390 bp) were amplified using FPY and R3Y as primers. Sequences encoding the putative binding-protein genes (*dppA* and *dppP*) were amplified with the primers FEY and REY (2.0 kb) and FEP and REP (2.2 kb), respectively. In these cases, a *Nco*I restriction site was introduced in the forward primers and a *Bam*HI restriction site in the reverse primers (Table 2). The PCR products were digested with *Nco*I and *Bam*HI and ligated with the 3.7-kb fragment that was obtained by digestion of pHLP5 (Putman et al. 1999) with the corresponding enzymes. This process was used to generate the vectors pNZAE (*dppA* gene) and pNZPE (*dppP* gene), which were subsequently analyzed by DNA sequencing (Table 1). *L. lactis* NZ9000 was used as host for these vectors. Sequences comprising the *dppB* and *dppC* genes were obtained using F1B and RMC1 as primers (2.1 kb). The primers FMC2 and RMC6 were used to amplify a 1.0-kb fragment containing the *dppD* gene and the 5′-end of *dppF*. The remaining sequence of *dppF* and the downstream region were amplified using FMC5 and RMC8 as primers (1.4 kb). DNA sequencing of either purified PCR products or plasmid vectors was carried out by the dideoxy-chain termination method (Sanger et al. 1977). Sequence-similarity searches were done with the FASTA algorithm of Pearson and Lipman (1988). Sequences were aligned with the program CLUSTAL W (version 1.8), after which the files were edited in GeneDoc (version 2.5.000).

Table 2 PCR amplification primers used in this study. Recognition sites of the restriction enzymes used are in boldface

Name	Sequence (restriction enzyme)
FPY	5′-GCAGATTTGAAACCAACTGACC-3′
R3Y	5′-TGCAACATCATAAGAATACTGG-3′
FEY	5′-CGCG**CCATGG**TATTTTAGTGGCCTGTGG-3′ (*Nco*I)
REY	5′-GC**GGATCC**AAGTCTTCATGTAATTTCCC-3′ (*Bam*HI)
FSY	5′-CGCG**CCATGG**AACAAGGAAAAATTATTGG-3′ (*Nco*I)
RFY	5′-GC**GGATC**CTTTAATATAAGCCGATTTTAAGTCG-3′ (*Bam*HI)
FEP	5′-CGCG**CCATGG**GTTTGTGAAAACTTTCCGC-3′ (*Nco*I)
REP	5′-GC**GGATCC**GGTTTATCTAAACCATAGGC-3′ (*Bam*HI)
F1P	5′-GGGAAATT**CCATGG**AGACTTGG-3′ (*Nco*I)
R1P	5′-GGC**GGATCC**TTTGAGGTAAGCTGTTTTG-3′ (*Bam*HI)
FWP	5′-CGCG**CCATGG**CAATAGCGGTTCTAGC-3′ (*Nco*I)
RXP	5′-GC**TCTAGA**TTTGAGGTAAGCCGTGC-3′ (*Xba*I)
F1B	5′-CGCGCCATGGTTAAATATATTCTTAAACG-3′
RMC1	5′-TTAAATCAAGAAGGTTACGCCC-3′
FMC2	5′-GTTGGTGAGTCTGGTTCTGG-3′
RMC6	5′-CAACGAGACCAAATGTTTCTCC-3′
FMC5	5′-GGAAACGCTGGGAAGAATTGAAAGG-3′
RMC8	5′-CAATAATTGTTCTGTAATCGTCGTCTTACC-3′
IFY	5′-CG**GGATCC**TCATCACTTGATACTTTAGG-3′ (*Bam*HI)
IRY	5′-CG**GAATTC**GTCTTTGTTTGAATGGAACG-3′ (*Eco*RI)
IFB	5′-CG**GGATCC**CCAACCAACCGGTTATTACG-3′ (*Bam*HI)
IRB	5′-CG**GAATTC**TTTTCAATCAAGACTGACCC-3′ (*Eco*RI)
IFF	5′-CG**GAATTC**TCTGGTAAAACAACGATTGG-3′ (*Eco*RI)
IRF	5′-CG**GGATCC**TCAGAGATAGGGTCTGGTGACG-3′ (*Bam*HI)
EM	5′-GTTTGGTTGATAATGAACTGTGC-3′
RF	5′-TAGAGTGGCATTTTTCTTATACACTTCC-3′

Amplified expression of the genes for the binding proteins

The *dppA* gene was amplified by PCR using the primers FSY and RFY and chromosomal DNA of *L. lactis* MG1363 as template. The *dppP* gene was amplified with the primers F1P and R1P using pNZPE as template. In both cases, *Nco*I and *Bam*HI restriction sites were introduced in the forward and reverse primers, respectively, allowing directional cloning of the PCR products in the expression vector pHLP5 (Putman et al. 1999). Thus, the binding-protein genes were placed under the control of the *nisA* promoter and in frame with the sequence specifying a carboxyl-terminal factor Xa cleavage site and a 6-histidine tag (Putman et al. 1999). The resulting vectors were designated pNZDppAL and pNZDppPL. The *dppP* gene was also amplified with the primers FWP and RXP containing *Nco*I and *Xba*I restriction sites. The PCR product was digested and ligated into the *Nco*I/*Xba*I restriction sites of pNZCIP (Bandell and Lolkema, University of Groningen, The Netherlands, unpublished data). The resulting vector, pNZDppP, carries the *dppP* gene, without its signal sequence, under the control of the *nisA* promoter and in frame with an amino-terminal factor Xa cleavage site and a 6-histidine tag. *L lactis* NZ9000 was used as host for the *nisA*-controlled expression vectors. The supernatant of cultures of *L. lactis* NZ9700 was used as a source of nisin A to trigger the expression of the binding-protein genes (Kuipers et al. 1993).

Construction of disruption mutants

Individual disruptions of *dppA*, *dppB*, and *dppF* were obtained by plasmid integration via homologous recombination (Leenhouts et al. 1989) using the double peptide-transport mutant *L. lactis* AG500 (Table 1). A 0.86-kb fragment (positions 661–1,522) of *dppA* was amplified by PCR with the primers IFY and IRY (Table 2) using pNZAE as template. A 0.5-kb fragment (positions 4,144–4,645) of *dppB* was amplified with the primers IFB and IRB (Table 2) using chromosomal DNA from *L. lactis* MG1363 as template. A 0.65-kb fragment (positions 7,057 to 7,709) of *dppF* was amplified with the primers IFF and IRF (Table 2) using chromosomal DNA from *L. lactis* MG1363 as template. In every case, restriction sites were introduced into the forward and reverse primers to allow directional cloning into the *Eco*RI/*Bam*HI sites of the integrative vector pORI28 (Em^r) (Leenhouts and Venema 1993). *L. lactis* LL108 was used as host of the new integration vectors (pINTdppA, pINTdppB and pINTdppF, Table 1) since this strain carries the *repA* gene, which is needed for replication of the plasmids, in trans. Subsequently, *L. lactis* AG500 was transformed with the new constructs (pINTdppA, pINTdppB, and pINTdppF) to inactivate the corresponding genes (*dppA*, *dppB*, and *dppF)*. Transformants resistant to erythromycin were selected, and the integration of each plasmid into the corresponding gene was verified by PCR amplification and Southern hybridization. The following primers were used for the PCR amplifications: EM and FSY to check the integration of pINTdppA; EM and F1B to check the integration of pINTdppB; EM and RF to check the integration of pINTdppF (Table 2). For Southern analysis, chromosomal DNA from putative integrant mutants was digested with the appropriate restriction enzymes, and the fragments were separated in 0.8% agarose gels. After electrophoresis, DNA was transferred to positively charged nylon membranes (BioRad, Veenendaal, The Netherlands) by Southern blotting. Probe labeling and hybridization were carried out using the non-radioactive DNA Labeling and Detection kit (Boehringer) according to the instructions of the manufacturer. The *dppA* disruption mutant was designated *L. lactis* YS5A, the *dppB* disruption mutant *L. lactis* YS5B, and the *dppF* disruption mutant *L. lactis* YS5F (Table 1).

Preparation of samples for the determination of protein-expression levels

The effect of different growth conditions on the expression level of DppA was determined in membrane fractions obtained as previously described (Sanz et al. 2000). The expression of the binding-protein genes (*dppA* and *dppP*), placed under the control of the *nisA* promoter, was determined in total cell lysates obtained by sonication of nisin-induced cells (Sanz et al. 2000). The effect of peptides in the growth medium on the expression levels of the proteinase (PrtP) and some peptidases (PepN, PepC, PepA and PepT) was also studied in total cell lysates obtained by sonication of cultures collected in the mid- and late-exponential phase of growth.

Electrophoresis and Western analysis

SDS-PAGE was carried out according to Laemmli (1970) using 6% acrylamide stacking gels and 10% acrylamide resolving gels. Proteins were visualized by Coomassie brilliant blue staining or transferred to polyvinylidene difluoride membranes (Boehringer) by semidry electroblotting (Kyhse-Anderson 1984). Monoclonal antibodies (Dianova) raised against the 6-histidine tag were used to detect the expression of DppA and DppP (Sanz et al. 2000). The effect of growth conditions on the expression of wild-type DppA was determined using polyclonal antibodies raised against the purified DppA-$(His)_6$ (Sanz et al. 2000). Monoclonal antibodies (anti-PrtP, -PepN, -PepC, -PepA and -PepT) were used at 8× dilutions. Primary antibodies were detected with the Western-Light chemiluminescence kit using CSPD as substrate (Tropix, Bedford, Mass.).

Protein concentration

The concentration of protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Nucleotide sequence accession number

The sequence data have been submitted to the GenBank database under accession number AF247635.

Results

Nucleotide-sequence analysis of *dpp* genes

Of all proteolytic systems involved in the breakdown of exogenous proteins such as caseins, the one in *L. lactis* subsp. *cremoris* MG1363 is the best characterized to date. Several dozens of isogenic mutants have been constructed in the MG1363 background, and therefore this strain was used to isolate the *dpp* genes. An 8.3-kb chromosomal DNA region of *L. lactis* MG1363 was isolated with the aid of the primers designed on the basis of the genome sequence of *L. lactis* subsp *lactis* IL1403. The DNA fragment comprises six ORFs, which are schematically depicted in Fig. 1. All ORFs are predicted to start with an ATG translation initiation codon and are preceded by putative ribosome-binding sites (ΔG_0 from –9.4 to 11.6 kcal/mol; Van de Guchte et al. 1992). ORF1 (DppA) contains 550 codons. Potential lactococcal –35 and –10 promoter sequences were found upstream of ORF1 (De Vos and Simons 1992). A 14-nucleotide inverted repeat (ΔG_0 of –11.6 kcal/mol), which could be used as a terminator, was found nine nucleotides downstream of the stop codon of ORF1. ORF2 (DppP) comprises 534 codons, but it is interrupted by a stop codon that is not present in the corresponding gene from the IL1403 strain. Potential lactococcal –35 and –10 promoter sequences were found upstream of ORF2. ORF3

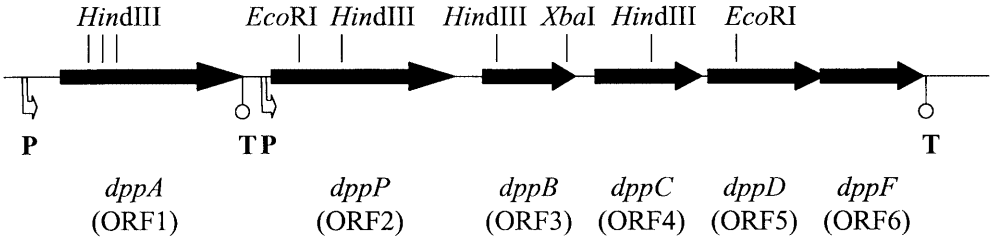

Fig. 1 The 8.3-kb chromosomal region encoding the Dpp dipeptide-transport system of *Lactococcus lactis* subsp. *cremoris* MG1363. *Arrows* represent the lengths and orientations of the genes. The positions of putative promoter (*P*) and terminator (*T*) elements are shown. *Bars* indicate relevant restriction sites

(DppB) and ORF4 (DppC) consist of 278 and 342 codons, respectively. ORF5 (DppD) and ORF6 (DppF) comprise 349 and 338 codons, respectively. The stop codon of ORF5 and the start codon of ORF6 overlap.

Table 3 Sequence comparison of Dpp proteins of *L. lactis* MG1363 and related ones of gram-negative and gram-positive bacteria. The percentage of identity between pairs of proteins was determined with the FASTA algorithm of Pearson and Lipman (1988)

	DppA	DppB	DppC	DppD	DppF
	Identity (%)				
L. lactis					
Opp	24.2	31.4	27.7	44.3	49.4
B. subtilis					
Dpp	29.7	41.2	39.0	53.9	43.2
SpoOK	30.6	44.4	37.5	62.5	60.6
E. coli					
Opp	28.9	35.4	38.3	50.9	49.1
Dpp	24.9	30.6	27.3	45.2	45.9
S. typhimurium					
Opp	30.3	35.6	36.5	51.9	49.3
H. influenzae					
Opp		37.1	37.8	50.6	50.0
Dpp		36.0	29.4	45.8	46.5

Deduced amino acid sequence and similarity searches

The protein sequences deduced from the ORFs identified in *L. lactis* MG1363 and their organization correspond with that of a binding-protein-dependent ABC transporter (Fig. 1). The protein specified by ORF1, designated DppA (57.7 kDa), shows the highest similarity with the pheromone-binding proteins PrgZ and TraC (31–32% identity) and the binding proteins of oligo- and di-tripeptide transport systems (Table 3). The sequence encompasses a putative N-terminal signal peptide of 22 residues, including a consensus lipo-modification site (LVACG). Translation of ORF2 will most likely result in a truncated protein due to the presence of a stop codon at position 2,893. Also, a frame-shift relative to the coding sequence of *L. lactis* IL1403 is found at position 3,658. ORF2 would specify for a second peptide-binding protein (DppP), but it might only be functional in *L. lactis* IL1403. The proteins specified by ORF3 (30.3 kDa) and OFR4 (37.4 kDa), designated DppB and DppC, respectively, share high similarity with the integral membrane components of ABC transporters (Table 3). DppB and DppC of *L. lactis* MG1363 contain six and five predicted transmembrane helices, respectively (data not shown). The proteins specified by ORF5 (38.9 kDa) and ORF6 (34.9 kDa), designated DppD and DppF, respectively, display high similarity with ATP-binding proteins, especially with those of the peptide-transport systems (Table 3). Highly conserved regions typical of ABC-type ATP-binding proteins, including the Walker motifs A, B, and C-motif (Bianchet et al. 1997; Walker et al. 1982), are also found in DppD and DppF of *L. lactis* MG1363 (Fig. 2).

Fig. 2 Alignment of part of the ATP-binding proteins of several peptide-transport systems (DppD and DppF of *L. lactis*, AmiF of *St. pneumoniae*, OppF of *E. coli*, DppD and OppD of *B. subtilis*), with the nucleotide-binding domains highlighted The two Walker motifs (Walker et al. 1982), A (*WA*) and B (*WB*), and the C motif (Bianchet et al. 1997) are *underlined*. Conserved residues are in *boldface*

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                                        WA
DppFLlac  (27-75)    AINNVSLDIYEGETFGLVGESGSGKTTIGRAILKLYDNF---ITGGEILFEG
AmiFSpneu (34-80)    AVNNVSFEVEKNKTLGLVGESGCGKSTTLRSIMQLYT-----PTSGNIYFNG
OppFEcoli (38-84)    AVDGVTLRLYEGETLGVVGESGCGKSTFARAIIGLVK-----ATDGHVAWLG
DppDLlac  (25-75)    AIRNVSFDLEKGQTLAIVGESGSGKSVTTKTLMGLNAKN-AEIPEGELLFKG
OppDBsub  (26-77)    AIRGVNFHLDKGETLAIVGESGSGKSVTSQAIMKLIPMPPGYFKRGEILFEG
DppDBsub  (23-74)    AVRGVSFDLYKGETFAIVGESGCGKSVTSQSIMGLLPPYSAKVTDGRILFKN

                                  C motif                WB
DppFLlac  (148-199) LTRYPHEFSGGQRQRIGIARALAVKPKFVVADEPISALDVSIQAQVVNLMRD
AmiFSpneu (82-157)  LTRYPHEFSGGQRQRIGIARALALNPKLLLLDEAVSALDVSIRAQILNLLKA
OppFEcoli (158-209) INRYPHEFSGGQCQRIGIARALILEPKLIICDEPVSALDVSIQAQVVNLLQQ
DppDLlac  (151-202) INDYPHQWSGGMRQRAVIAIALAADPEILIADEPTTALDVTIQAQIMHMMAE
OppDBsub  (152-203) VNQFPHEFSGGMRQRVVIAMALAANPKLLIADEPTTALDVTIQAQILELMKD
DppDBsub  (148-200) LKQYPHQFSGGMRQRIVIAMALICEPDILIADEPTTALDVTIQAQILELFKE
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Localization and expression of DppA

Several expression vectors were constructed to prove whether or not ORF1 and ORF2 specify substrate-binding proteins of the Dpp system. Expression of the binding protein encoded by *dppA* of *L. lactis* MG1363 was achieved using vector pNZDppAL, which carries the *nisA* promoter and ribosome-binding site. A protein band of the expected size (approx. 57.7 kDa) was detected only in the membrane fraction (data not shown). This result is consistent with DppA being associated with the membrane via the predicted lipid modification of the N-terminus. Expression of *dppP* of *L. lactis* MG1363 was not observed, which is in accordance with the stop codon identified in its coding sequence.

Expression of DppA

The expression levels of DppA were quantified, after isolation of membrane fractions, using polyclonal antibodies raised against purified DppA (Sanz et al. 2000). *L. lactis* MG1363 (wild-type) and isogenic peptide transport mutants (AG300 [DtpT$^-$], AG500 [DtpT$^-$, Opp$^-$] and YS5B [DtpT$^-$, Opp$^-$, DppB$^-$]) were grown in M17, a peptide-rich medium, and CDM containing only free amino acids as nitrogen source. In M17, the expression of DppA was maximal in the double mutant *L. lactis* AG500 (DtpT$^-$, Opp$^-$), while it was reduced to 30% in the single mutant *L. lactis* AG300 (DtpT$^-$) and not detected in the wild-type strain (data not shown). The differences in expression levels were not observed when cells were grown in CDM (Fig. 3A). The expression of DppA in AG500 (DtpT$^-$, Opp$^-$) grown in CDM was about two-fold higher than in M17 (data not shown). Since transcriptional regulation of components of the proteolytic system seems to be dependent on particular peptides, i.e., those containing branched-chain amino acids (Guédon and Renault, INRA, Jouy-en-Josas, unpublished data), the expression of DppA in *L. lactis* AG500 (DtpT$^-$, Opp$^-$) was also studied in CDM in which a given amino acid was present in the form of a di- or tripeptide (Fig. 3A and Fig. 3B). For these assays, several peptides with different composition and binding affinity to DppA (Leu-Leu, Val-Val, Arg-Arg, Leu-Leu-Leu, Val-Val-Val, Ala-Ala-Ala as high-affinity and His-Gly as low-affinity substrate; Sanz et al. 2000) were tested. Growth on tri-leucine or tri-valine as the only source of leucine or valine, respectively, resulted in a drastic reduction in DppA expression in *L. lactis* AG500 (Fig. 3A, B). In contrast, growth on tri-leucine together with leucine had no effect on DppA expression (Fig. 3B). Moreover, the other peptides tested (His-Gly, Arg-Arg, Ala-Ala-Ala, Leu-Leu and Val-Val) did not repress significantly (Fig. 3A, B). Taken together, the data indicate that down-regulation of DppA expression depends on the transport of certain hydrophobic tripeptides, but other factors, such as high intracellular amino acid (leucine) pools, seem to exert anadditional level of control that overrides the repression by peptides. The down-regulation of DppA expression at mil-

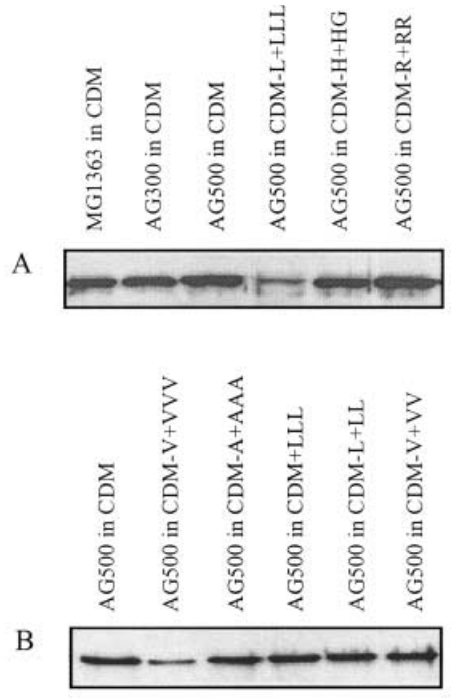

Fig. 3A, B Expression of DppA in *L. lactis* (wild-type and peptide-transport mutants) grown with different sources of amino acids. Proteins from membrane fractions (3.5 μg unless specified otherwise) were separated by SDS-(10%) PAGE and analyzed by immunoblotting using antiserum raised against purified DppA. Peptides were added as sources of the corresponding free amino acids at the following concentrations: **A** 1.2 mM Leu-Leu-Leu, 0.7 mM His-Gly, 0.35 mM Arg-Arg; **B** 0.9 mM Val-Val-Val, 0.9 mM Ala-Ala-Ala, 1.2 mM Leu-Leu-Leu, 1.8 mM Leu-Leu, 1.4 mM Val-Val.The concentrations of peptides reflect the concentrations of the individual amino acids present in chemically defined medium (CDM)

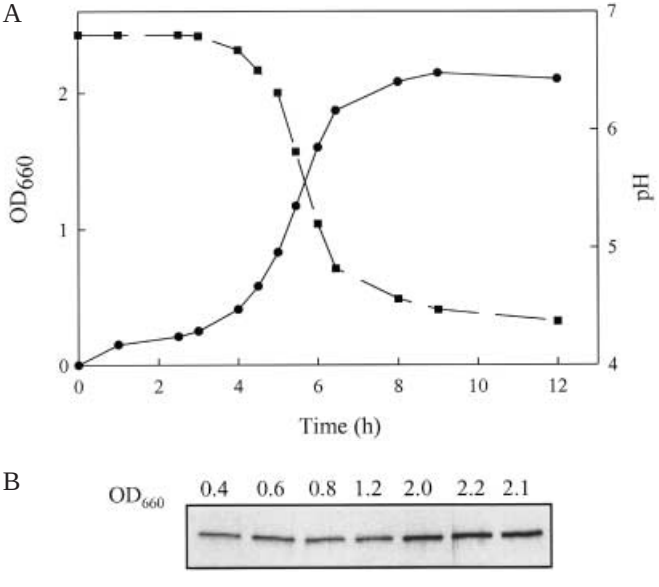

Fig. 4 Growth-phase-dependent expression of DppA in *L. lactis* AG500 grown in CDM. The growth curve (OD at 660 nm, l) and pH-values of the medium (n) are shown (**A**). The expression levels of DppA were quantified by Western analysis (**B**). Proteins of membrane fractions (3.5 μg) were separated by SDS-(10%) PAGE and analyzed by immunoblotting using antiserum raised against purified DppA

limolar concentrations of Val-Val-Val or Leu-Leu-Leu did not affect the growth rate of the strains (data not shown). DppA in *L. lactis* AG500 (DtpT$^-$, Opp$^-$) also showed growth-phase-dependent expression in CDM, being maximal in the stationary phase when the external pH reaches a minimum value (Fig. 4).

Function of *dpp* genes in di-tripeptide utilization

To establish the functionality of the *dpp* genes, *dppA*, *dppB*, and *dppF* were inactivated individually in *L. lactis* AG500 (DtpT$^-$, Opp$^-$). The chromosomal integration of a single copy of each of the gene-disruption plasmids was verified by Southern analysis. The phenotype of the mutants was inferred from their ability to utilize peptides. Growth experiments were carried out in CDM supplemented with erythromycin to avoid possible differences in growth rates due to the presence or the absence of the antibiotic. For this purpose, the strains used as controls, MG1363 (wild type) and AG500 (DtpT$^-$, Opp$^-$), were transformed with the empty vector pAMP0 carrying the erythromycin resistance gene; the corresponding strains were designated *L. lactis* MGE and AG5E, respectively (Table 1). Control experiments in CDM medium plus 5 μg/ml of erythromycin excluded the possibility that differences in growth characteristics were due to variations in copy number of the resistance marker gene in the strains used.

Growth rates were determined over a range of concentrations of di-valine (0.210–0.035 mM), representing between 15 and 2.5% of the requirements for optimal growth (Table 4). *L. lactis* AG5E (DtpT$^-$, Opp$^-$) and *dpp* disruption mutants (DtpT$^-$, Opp$^-$, Dpp$^-$) showed similar growth rates (about μ=0.3 h^{-1}) in the presence of 0.21 mM Val-Val. The wild-type strain MGE grew better (μ=0.82 h^{-1}) at these di-valine concentrations. Differences between the double (AG5E [DtpT$^-$, Opp$^-$]) and triple (YS5A [DtpT$^-$, Opp$^-$, DppA$^-$], YS5B [DtpT$^-$, Opp$^-$, DppB$^-$], and YS5F [DtpT$^-$, Opp$^-$, DppF$^-$]) mutants were observed when the Val-Val concentration was reduced to 0.140 mM or below. At 0.07 mM Val-Val, the three *dpp* disruption mutants (DtpT$^-$, Opp$^-$, Dpp$^-$) failed to grow, whereas the wild-type and double-mutant strains (DtpT$^-$, Opp$^-$) grew with μ values of 0.44 and 0.18 h^{-1}, respectively. The disruption of *dppA*, *dppB*, or *dppF* had no apparent effect on growth in CDM containing equivalent amounts of free valine (data not shown). The lowest concentration of di-valine tested was not sufficient to promote growth of any but the wild-type strain. These results indicate that *dppA* and *dppF* are essential for growth on micromolar concentrations of Val-Val. The disruption of *dppB* also impaired growth on low concentrations of Val-Val, but we cannot entirely rule out that (part of) this phenotype is due to polar effects on the downstream genes. The growth rates on some other di- and tripeptides were not significantly different between *L. lactis* AG5E (DtpT$^-$, Opp$^-$) and the *dpp*-disruption mutants (DtpT$^-$, Opp$^-$, Dpp$^-$, data not shown). These results indicate that an additional di- and tripeptide transport system must be functional in *L. lactis* MG1363.

Expression of the major components of the proteolytic system is regulated by dipeptide internalized via Dpp

The uptake of peptides in *L. lactis* MG1363 has been related to regulation of the expression of the extracellular proteinase PrtP and some intracellular peptidases (Marugg et al. 1995; Meijer et al. 1996). The role of the Dpp transport system, which should mediate the internalization of di-valine, in the regulation of proteolytic activity was studied in CDM. For these assays, *L. lactis* AG500 (DtpT$^-$, Opp$^-$) carrying the empty vector pAMP0 (*L. lactis* AG5E) and the *dppB*-disruption mutant (*L. lactis* YS5B [DtpT$^-$, Opp$^-$, DppB$^-$]) were transformed with the plasmid pLP712, which contains the *prtP* gene; the corresponding strains are designated *L. lactis* AG5EP and YS5BP, respectively (Table 1). The expression of PrtP, PepN, and PepC in *L. lactis* AG5EP (DtpT$^-$, Opp$^-$) and *L. lactis* YS5BP (DtpT$^-$, Opp$^-$, DppB$^-$) was initially studied at two stages of growth (OD_{660} of about 0.7 and 1.6) using CDM as the growth medium. In every case, the expression levels were maximal in the late-exponential phase of growth and were similar for both strains (data not shown). The effects on proteinase expression ofpeptides present in the medium were examined in cells collected in the late-exponential phase of growth. When *L. lactis* AG5EP (DtpT$^-$, Opp$^-$) was grown on CDM in which valine (2.8 mM) was replaced by an equivalent amount of Val-Val (1.4 mM), the expression of PrtP, PepC, and PepN was significantly reduced (Fig. 5). The repressing effect of di-valine was no longer observed when *dppB* was inactivated (Fig. 5). The expression levels of PrtP and PepC in *L. lactis* AG5EP (DtpT$^-$, Opp$^-$) were less than 50% of those found in

Table 4 Growth rates on di-valine as sole source of the essential amino acid valine. Growth rates were determined in CDM without valine but in the presence of di-valine (see Materials and methods section for details). Data are the mean of four replicates and the standard deviation is given. –No significant growth

Strains of *L. lactis*	μ (h^{-1}) Di-valine (mM)			
	0.210	0.140	0.07	0.035
MGE (wild-type)	0.82±0.08	0.67±0.09	0.44±0.04	0.24±0.05
AG5E (Opp$^-$, DtpT$^-$)	0.33±0.04	0.23±0.03	0.18±0.03	–
YS5A (Opp$^-$, DtpT$^-$, DppA$^-$)	0.31±0.05	0.17±0.04	–	–
YS5B (Opp$^-$, DtpT$^-$, DppB$^-$)	0.32±0.06	0.18±0.03	–	–
YS5F (Opp$^-$, DtpT$^-$, DppF$^-$)	0.31±0.03	0.18±0.03	–	–

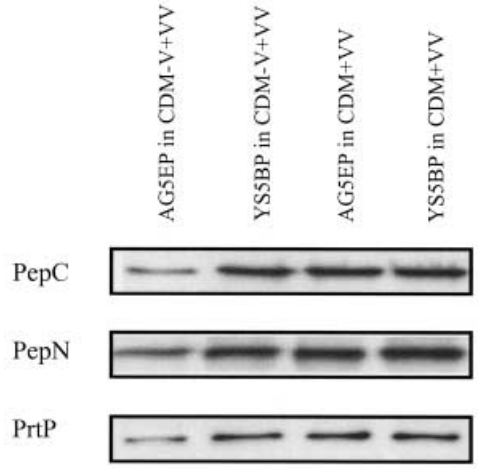

Fig. 5 Expression level of the peptidases C (PepC) and N (PepN), and the extracellular proteinase (PrtP) in *L. lactis* AG5EP (Opp$^-$, DtpT$^-$) and YS5BP (Opp$^-$, DtpT$^-$, DppB$^-$) during growth in either complete CDM plus 1.4 mM di-valine or CDM lacking valine but containing 1.4 mM di-valine. Proteins from total cell lysates were separated by SDS-(10%) PAGE and analyzed by immunoblotting using monoclonal antibodies raised against PrtP, PepC, or PepN. Equal amounts of total protein were loaded (2 μg per lane)

L. lactis YS5BP (DtpT$^-$, Opp$^-$, DppB$^-$). The expression level of PepN in *L. lactis* AG5EP (DtpT$^-$, Opp$^-$) was reduced by about 30% compared to that in *L. lactis* YS5BP (DtpT$^-$, Opp$^-$, DppB$^-$). When both freevaline and the dipeptide Val-Val were added to the growth medium, the differences in expression were no longer observed (data not shown). Thus, similar to the regulation of expression of DppA, free amino acids counteract the down-regulation of the proteolytic enzymes by hydrophobic peptides.

Discussion

In this work, we describe the genetic organization of a new peptide transport system (Dpp) of *L. lactis* subsp. *cremoris* MG1363. Evidence for its role in dipeptide utilization and regulation of the central components of the proteolytic system is presented.

Sequence comparisons demonstrate that the *dppA* and *dppPBCDF* operons of *L. lactis* specify a binding-protein-dependent ABC transporter. The presence of more than one gene specifying bindingproteins that serve the same membrane complex is not unusual among ABC transporters. In gram-positive bacteria, such as *S. pneumoniae*, several genes, present at different locations on the chromosome, specify binding proteins that interact with the membrane complex encoded by the *ami* locus (Alloing et al. 1994). The *dppA* and *dppP* genes are closely related to each other, suggesting that they resulted from recent gene duplication. The mutations in *dppP* of the strain MG1363 may have not been selected against because of a redundancy of systems involved in dipeptide uptake in *L. lactis*.

The predicted amino acid sequence of DppA includes an N-terminal signal peptide with the signature typical of lipoproteins of gram-positive bacteria (Tam and Saier 1993). The expression of DppA with and without the signal peptide, and its localization in the membrane and cytoplasmic fractions, respectively, confirms that the signal peptide targets the protein to the cytoplasmic membrane (Sanz et al. 2000). The substrate-binding proteins provide the primary interaction site for the ligand and largely define the specificity of the transport system (Sleigh et al. 1997). The kinetics and specificity of purified DppA as a high affinity di- and tripeptide-binding protein were previously reported (Sanz et al. 2000). Peptides consisting of basic and hydrophobic residues bind to DppA with the highest affinity, but italso accepts hydrophilic neutral and acidic peptides. Although DppP is not functional in the MG1363 strain, the equivalent protein in IL1403 is capable of binding di- and tripeptides. Preliminary experiments indicate that DppP of IL1403 binds hydrophobicand basic peptides with nanomolar dissociation constants (K_d), whereas Gly-Gly and Glu-Glu are bound with K_d values in the millimolar range (Sanz et al., IATA (C.S.I.C), unpublished results). These data suggest that there are no large differences in binding activity of DppA and DppP.

The ATP-binding proteins DppD and DppF have the highest similarity with the corresponding components of other bacterial species. The stop codon of DppD and the start codon of DppF overlap, suggesting that these two genes are translationally coupled. Such coupling would ensure that their products, which most likely function as an ATP-binding heterodimer, are synthesized in equal amounts (Podbielski and Leonard 1998).

The absence of growth of the *dppA*, *dppB*, and *dppF* mutants at low concentrations of di-valine is consistent with the observations that the gene products constitute a functional peptide-transport system. The differences in growth rates on di-valine were only detected at low concentrations of the dipeptide, a resultconsistent with the high affinity of the system (Sanz et al. 2000). Furthermore, the absence of differences in growth rates on other di- or tripeptides suggests the existence of a fourth peptide-transport system whose specificity overlaps that of Dpp, which was not anticipated at the start of this work (Foucaud et al. 1995).

The expression of the DppA binding protein of *L. lactis* is regulated by the peptide content of the growth media, an effect deduced from the different levels of DppA present in cells grown on CDM, with amino acids as sole source of nitrogen, and M17, a complex medium containing various peptides. The expression of DppA also depends on the presence or absence of other peptide-transport systems (Opp and/or DtpT), indicating that the peptides need to be taken up in order to exert their regulatory effect. The expression of DppA in cells grown in peptide-rich medium is highest in the mutant lacking both Opp and DtpT, which suggests that expression of DppA can be used to compensate for deficiencies in other peptide transporters. The effect of specific peptides on the expression of DppA shows that, of all peptides tested, only tripeptides composed of branched-chain residues (leucine and valine) down-regulate the expression of DppA. However, the combination of either of these tripeptides and the corresponding free amino

acid did not provoke the repressing effect. Therefore, not only the hydrophobic tripeptides constitute a signal but also high levels of free amino acids lead to a response. There is no obvious rationale as to why amino acids should overcome the repression by tripeptides.

In *L. lactis*, the expression of DtpT and Opp is also regulated by the peptide content of the media. The expression of DtpT is three to five times higher in CDM than in complex medium containing peptides (Hagting et al. 1997). Expression of the OppA binding protein of the oligopeptide transport system is also ten-fold higher in CDM than inpeptide-rich broth (Detmers et al. 1998). Furthermore, the residual uptake activity of mutants lacking both Opp and DtpT is stimulated about three-fold in CDM (Foucaud et al. 1995). The picture that emerges from these studies is that particular peptides down-regulate the expression of the various peptide-transport systems and that maximal expression is observed when only amino acids are present in the medium. In the case of DppA, the free amino acids even abolish the repressing effect of Leu-Leu-Leu, which may relate to high cytoplasmic levels of Leu that build up when these amino acids are present in the medium. Since the expression levels of DppA in media containing peptides depends on whether or not other peptide-transport systems are present, it is most likely that the "regulatory" peptides need to be internalized and that the intracellular levels of peptides are sensed. Finally, a consequence of the regulatory mechanism is that the peptide transport capacity is maximal when the availability of peptides is limiting. The cell is then able to scavenge any peptide that it encounters in the environment. The cell lowers the peptide transport capacity when peptide availability is high, perhaps because excessive cellular levels of peptides interfere with biosynthesis (Chopin 1993). The observation that DppA is maximally expressed in the stationary phase of growth is also consistent with a role in peptide scavenging, that is, under these conditions the cell densities are high and the pH and nutrient concentrations are low. The very high affinity of DppA for the majority of peptides, even at the low pH values typical for the stationary phase of growth, allows *L. lactis* to efficiently capture low peptide concentrations.

Repression of transcription of the *dpp* operon in *E. coli* and a group A streptococcal strain by peptides has also been described, but, in most experiments, only complex peptide (and amino acid) mixtures are used to study the effect of nitrogen availability (Olson et al. 1991; Podbielski and Leonard 1998). In the streptococcal strain, the *dppA*-specific transcript is more abundant than the polycistronic *dpp* mRNA, and the ratio of *dppA* to *dpp* decreases in the presence of dipeptides (Podbielski and Leonard 1998). The nitrogen source has also been shown to affect the expression of the *dpp* operon in *B. subtilis*, but the effects are thought to be growth rate-related rather than nitrogen source-specific (Serror and Sonenshein 1996). We now show for *L. lactis* that regulation of expression of DppA is peptide-specific, i.e., repression is only observed with tripeptides composed of branched-chainamino acids. To our knowledge, the dichotomy in the regulation by di- and tripeptides has not been reported before, not for *L. lactis*, *B. subtilis*, *E. coli* or other species. The regulation of expression of the peptide transporters Opp and DtpT, and the proteolytic enzymes PrtP, PepN and PepC, in *L. lactis* MG1363 is also under peptide control, but with these systems di- and tripeptide seem to exert similar effects (Detmers et al. 1998; Hagting et al. 1997; Marugg et al. 1995; Meijer et al. 1996).

The role of *dpp* in the control of the proteinase and peptidase components of the proteolytic system of *L. lactis* MG1363 occurs most likely via the internalization of peptides. Di-valine, when provided as sole source of valine, exerts a repressing effect on the expression of PrtP, PepN, and PepC that is no longer observed when *dppB* is inactivated. Consistent with these findings are studies on the transcriptional regulation of genes encoding different components of the proteolytic system by di- and tripeptides containing branched-chain amino acid residues (Leu, Val, and Ile; Guédon et al., INRA, Jouy-en-Josas, unpublished data). However, the precise mechanism of regulation and the full complement of molecules that participate in the signaling process remain to be identified. Nevertheless, the data point towards a role for intact intracellular peptides and their degradation products (free amino acids) in the regulation of expression of the various components of the proteolytic system. Peptides and free amino acids seem to exert opposite effects on the expression of the components of the proteolytic system of *L. lactis* studied thus far.

Acknowledgements This work was supported by the European Union (BIO-4-CT-960016). Y. Sanz was a postdoctoral fellow of the Ministerio de Educación y Cultura (Spain).

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